

TITLE OF THE INVENTION

Low Temperature Expression Chitinase cDNAs
and Method for Isolating the Same

BACKGROUND OF THE INVENTION

5 The present invention relates to chitinase cDNAs and to a method for their isolation, and more specifically it relates to chitinase cDNAs having a function of conferring plant disease resistance under low temperature, and to a method of isolating the chitinase cDNAs.

10 In the northern regions, overwintering crops such as barley, forage grasses and wheat must survive subzero temperature (0 °C or below 0 °C) and a long-lasting snow cover condition (0 °C in darkness). However, overwintering crops in such environment are often attacked by snow molds which are a diverse group of psychrophilic parasitic fungi. This biotic stress greatly limits yields and quality of biennial or perennial crops, in the same manner as a low temperature stress will do in the northern region with snow accumulation.

15 In current winter wheat cultivation, it is necessary to apply a broad-spectrum fungicides before a continuous snow cover for protecting the plant from snow molds infection.

20 However, it has taken high cost and it has been proved difficult to apply the fungicide at the effective time, because of unstable nature of the start of a snow cover every year.

In view of the above, it has been desired to raise a plant variety having a high disease resistance under low temperature environment.

Nevertheless, up till now, when using several conventional breeding methods each based on cross-breeding, it has not been possible to raise superior varieties with high resistance, and a long time (many years) is required for raising superior varieties. For this reason, there has been a strong demand for variety improvement by more effective methods such as gene engineering methods.

As a result of repeated diligent research over years aimed at solving the problems described above, the inventors of the present invention have arrived at the following conclusion. Specifically, it has been found that plant disease resistance under low temperature environment is induced by cold acclimation that occurs under a low temperature from autumn through winter (hereunder referred to as "hardening") and that expression of the three chitinase cDNAs of the invention described hereunder are found during this hardening, with the translation product conferring plant disease resistance through digestion of chitin, one of the major components of fungus cell wall.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide chitinase cDNAs that encode proteins having enzymatic

function in low temperature environments and that when introduced into plants confer plant disease resistance.

It is another object of the invention to provide a method for isolation of chitinase cDNAs that encode proteins having enzymatic function in low temperature environments and that when introduced into plants confer plant disease resistance.

According to one aspect of the present invention, there is provided a winter wheat-derived chitinase cDNA, characterized in that said cDNA has a nucleotide sequence corresponding to an amino acid sequence listed as SEQ.ID. No.1 in Fig. 1. In detail, said cDNA comprises 771 nucleotides/256 amino acids and has 98% identity (on amino acid sequence level) with barley-derived chitinase cDNA. In more detail, said cDNA encodes a protein with chitinase activity in low temperature environment and confers plant disease resistance by digestion of chitin, one of the major components of fungus cell wall.

According to another aspect of the present invention, there is provided another winter wheat-derived chitinase cDNA, characterized in that said cDNA has a nucleotide sequence corresponding to an amino acid sequence listed as SEQ.ID. No.2 in Fig. 2. In detail, said cDNA comprises 972 nucleotides/323 amino acids and has 68% identity (on amino acid sequence level) with rye-derived chitinase cDNA. In more detail, said cDNA encodes a protein with chitinase activity in low temperature environment and confers plant

disease resistance by digestion of chitin, one of the major components of fungus cell wall.

According to a further aspect of the present invention, there is provided a further winter wheat-derived chitinase cDNA, characterized in that said cDNA has a nucleotide sequence corresponding to an amino acid sequence listed as SEQ.ID. No.3 in Fig. 3. In detail, said cDNA comprises 960 nucleotides/319 amino acids and has 95% identity (on amino acid sequence level) with spring wheat-derived chitinase cDNA. In more detail, said cDNA encodes a protein with chitinase activity in low temperature environment and confers plant disease resistance by digestion of chitin, one of the major components of fungus cell wall.

According to a still further aspect of the present invention, there is provided a method of isolating a winter wheat-derived chitinase cDNA having a nucleotide sequence corresponding to an amino acid sequence listed as SEQ.ID. No.1 in Fig. 1, a winter wheat-derived chitinase cDNA having a nucleotide sequence corresponding to an amino acid sequence listed as SEQ.ID. No.2 in Fig. 2, a winter wheat-derived chitinase cDNA having a nucleotide sequence corresponding to an amino acid sequence listed as SEQ.ID. No.3 in Fig. 3, said method comprising the steps of: extracting mRNA from winter wheat variety PI173438 (having high snow molds resistance) that has undergone a sufficient hardening process; preparing cDNA and a cDNA library based on said mRNA; analyzing

nucleotide sequences of a number of plant-derived chitinase cDNAs which have all been published by EMBL/Genebank/DDBJ DNA Databank; designing a pair of chitinase cDNA-specific degenerated primers with reference to highly conserved nucleotide sequence portions of the plant-derived chitinase cDNAs; conducting PCR (polymerase chain reaction) using a pair of chitinase cDNA-specific degenerated primers and using said cDNA as a template, thereby amplifying fragments of chitinase cDNAs and obtaining amplified DNA fragments; and using said amplified DNA fragments as probes for screening said cDNA library by a hybridization assay, to isolate recombinant plaques containing full length of cDNA.

In particular, one of the pair of chitinase cDNA-specific degenerated primers has the following nucleotide sequence:

(Forward): 5' C-A-C-G-A-G-A-C-C-A-C-N-G-G-C-G-G-N-T-G-G-G-C
(SEQ. ID. No. 4),

and the other has the following nucleotide sequence:

(Reverse): 5' A-C-N-A-A-T-A-T-C-A-T-C-A-A-C-G-G-C-G-G
(SEQ. ID. No. 5).

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows an amino acid sequence of SEQ. ID No. 1.

Fig. 2 shows an amino acid sequence of SEQ. ID No. 2.

Fig. 3 shows an amino acid sequence of SEQ. ID No. 3.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The cDNAs of the present invention are chitinase cDNAs capable of expressing under a low temperature condition.

The method for isolating the cDNAs of the present invention may be carried out in the following manner.

5 Specifically, mRNA is extracted from winter wheat PI173438 (having high snow molds resistance) that has undergone a hardening process (low temperature acclimation) under natural conditions in Sapporo City, Japan until November 22. This mRNA is then used to prepare cDNA and a cDNA library.

10 Next, nucleotide sequences of a number of plant-derived chitinase cDNAs which have all been published by EMBL/Genbank/DDBJ DNA Databank are closely analyzed, and a pair of chitinase cDNA-specific degenerated primers are designed with reference to highly conserved nucleotide sequence portions.

15 The pair of designed chitinase cDNA-specific degenerated primers are used in a PCR (polymerase chain reaction) using the above-mentioned cDNA as the template for amplifying the expected chitinase cDNA fragments (all are approximately 400 bp), and the amplified fragments are isolated.

20 The amplified fragments are used as probes for screening the cDNA library by a hybridization assay, to isolate recombinant plaques containing full length of cDNA. The nucleotide sequences of the isolated plaques were analyzed and demonstrated to be three different chitinase cDNAs which are

three kinds of chitinase cDNA fragments, all are novel in plants.

An example of the method for isolating the cDNAs of the present invention was carried out in the following steps 1) - 3).

1) Preparation of cDNA and cDNA library from
snow molds resistant winter wheat variety PI173438

mRNA was extracted by a common method from the crown portion of winter wheat (*Triticum aestivum* L.) PI173438 (having high snow molds resistance) that had been seeded in a container in late September and had then undergone a hardening process under natural conditions until November 22. A portion (5 μ g) of the obtained mRNA was used to synthesize cDNA utilizing a cDNA Synthesis Kit (STRATAGENE Co.). After attaching adaptors to both ends of the cDNA, it was incorporated into a ZAP Expression Vector (STRATAGENE Co.), thereby obtaining a cDNA library of approximately 6×10^6 pfu.

2) PCR using a pair of cDNA-specific degenerated
primers and using the cDNA as a template

One of the pair of chitinase cDNA-specific degenerated primers, having the following nucleotide sequence:

(Forward): 5' C-A-C-G-A-G-A-C-C-A-C-N-G-G-C-G-G-N-T-G-G-G-C
(SEQ. ID. No. 4),

the other chitinase cDNA-specific degenerated primer, having the following nucleotide sequence:

(Reverse): 5' A-C-N-A-A-T-A-T-C-A-T-C-A-A-C-G-G-C-G-G

(SEQ.ID. No.5).

which were synthesized based on highly conserved regions of the nucleotide sequences of known chitinase cDNAs (published by EMBL/Genebank/DDBJ DNA Databank), were used in a PCR using the cDNA (synthesized in the manner described in the above) as the template.

The PCR was performed in a final volume of 50 μ l. In detail, 1 μ l of Taq DNA polymerase (5 units/ μ l) by Nippon Gene Co., 5 μ l of 10 x PCR buffer (containing $MgCl_2$), 5 μ l of dNTP solution (10 mM), 2 μ l of each primer (12 μ M) and about 10 ng of the cDNA synthesized in the above, were mixed and then brought to a total of 50 μ l with distilled water. The PCR conditions and number of reaction cycles are shown in Table 1 below.

Table 1

PCR condition and number of reaction cycles

Initial Denaturation	94° C	1 min	once
Denaturation	94° C	1 min	30 cycles
Annealing	48° C	1 min	
Primer Extension	72° C	1 min	
Final Extension	72° C	2 min	once

(In Table 1, "denaturation" refers to a reaction in which double-stranded DNA is melt into single strand and secondary structure is eliminated, "primer extension" refers to an synthesizing of the new complementary strand, and "30 cycles" means that three basic steps of denaturation-annealing-primer extension are repeated with 30 cycles.

As a result, DNA fragments (having expected length of approximately 400 bp) of chitinase cDNAs were amplified by the above PCR with the pair of chitinase cDNA-specific degenerated primer having nucleotide sequence of SEQ.ID No.4 and the primer with the nucleotide sequence of SEQ.ID No.5. Theses amplified DNA fragments were then isolated and subsequently sequenced using a DNA sequencer (Model 373S by ABI Co.) according to the conventional method. By comparing the

sequences with known chitinase, it were confirmed that novel chitinase cDNA fragments (having a high homology with known chitinase cDNA) were isolated.

5 3) Isolation and nucleotide sequencing of full length
 cDNAs encoding chitinase of the present invention

 About 1×10^5 recombinant plaques from the cDNA library
obtained in the manner described in the above were subjected
to a hybridization assay by using filters lifted with 1×10^5
10 recombinant plaques, and using probes prepared by labeling
(with ^{32}P) each novel chitinase cDNA fragment obtained in the
above.

 The hybridization reaction was carried out for 16 hours
at 42°C , in a solution containing 50% formamide, 5 x SSPE, 5
15 x Denhardt's solution, 0.5% SDS and 0.2 mg/ml salmon sperm DNA
with ^{32}P -labeled probe.

 The filters were then washed twice in a solution
containing 2 x SSC and 0.1% SDS at 65°C for 10 min.
Afterwards, the filters were washed twice with another washing
20 solution containing 0.1 x SSC and 0.1% SDS, at 65°C for 15
min. Detection of each positive plaque binding to ^{32}P -labeled
probe was performed by exposing above washed filters to X-ray
films.

 About 45 positive recombinant plaques obtained in the
25 above were subjected to nucleotide sequencing with DNA
sequencer by ABI Co.

Analysis of the nucleotide sequences of these recombinant
plaques revealed that novel chitinase cDNAs having nucleotide
sequences corresponding to the amino acid sequences listed as
SEQ.ID Nos. 1 - 3 in Figs. 1 - 3 had been isolated from winter
wheat variety PI173438.

In fact, what were isolated were i) a novel winter wheat-
derived chitinase cDNA having a nucleotide sequence
corresponding to the amino acid sequence listed as SEQ.ID.
No.1 in Fig. 1, comprising 771 nucleotides/256 amino acids and
having 98% identity (on amino acid sequence level) with
barley-derived chitinase cDNA, ii) a novel winter wheat-
derived chitinase cDNA having a nucleotide sequence
corresponding to the amino acid sequence listed as SEQ.ID.
No.2 in Fig. 2, comprising 972 nucleotides/323 amino acids and
having 68% identity (on amino acid sequence level) with rye-
derived chitinase cDNA, iii) a novel winter wheat-derived
chitinase cDNA having a nucleotide sequence corresponding to
the amino acid sequence listed as SEQ.ID. No. 3 in Fig. 3,
comprising 960 nucleotides/319 amino acids and having 95%
identity (on amino acid sequence level) with spring wheat-
derived chitinase cDNA.

Investigation of Enzymatic Activity

In order to investigate enzymatic activities of the
novel chitinase cDNAs of the present invention, enzymatic
reactions were conducted under the following conditions using

culture solutions containing novel proteins secreted by recombinant yeast (into which each novel chitinase cDNA of the present invention has been introduced).

[Enzymatic Reaction Condition]

5 Buffer solution (20 mM citric acid/phosphoric acid), pH 4.5

Final substrate concentration: 1% collidal chitin

Reaction temperature: 38 °C, reaction time: 16 hours.

As a result, it was confirmed that the culture solutions containing novel proteins secreted by recombinant yeast (into which each novel chitinase cDNA of the present invention has been introduced) had a chitinase activity capable of producing a disaccharide (a chito-oligosaccharide) or a trisaccharide (another chito-oligosaccharide) from chitin polymer (serving as a substrate).

15 The nucleotide sequences of the novel cDNAs obtained in the present invention are listed in the following.

20

25

Nucleotide Sequence of cDNA corresponding to the

B Amino Acid Sequence Listed as SEQ. ID. No. 1 (SEQ ID NO: 6)

10	20	30	40	50	60
ATGGCGAGGT	TTGCTGCCCT	CGCCGTGTGC	GCCGCCGCGC	TCCTGCTCGC	CGTGGCGGCG
70	80	90	100	110	120
GGGGGTGCCG	CGGCGCAGGG	CGTGGGCTCG	GTCATCACGC	GGTCGGTGTA	CGCGAGCATG
130	140	150	160	170	180
CTGCCCAACC	GCGACAATC	GCTGTGCCCC	GCCAGAGGGT	TCTACACGTA	CGACGCCTTC
190	200	210	220	230	240
ATCGCCGCCG	CCAACACCTT	CCCGGGCTTC	GGCACCACCG	GCAGCGCCGA	CGACATCAAG
250	260	270	280	290	300
CGCGACCTCG	CCGCCTTCTT	CGGCCAGACC	TCCACAGAGA	CCACCGGAGG	GACGAGAGGC
310	320	330	340	350	360
GCTGCCGACC	AGTTCCAGTG	GGGCTACTGC	TTCAAGGAAG	AGATAAGCAA	GGCCACGTCC
370	380	390	400	410	420
CCACCATACT	ATGGACGGGG	ACCCATCCAA	TTGACAGGGC	GGTCCAACTA	CGATCTTGCC
430	440	450	460	470	480
GGGAGAGCGA	TCGGGAAGGA	CCTGGTGAGC	AACCCAGACC	TAGTGTCCAC	GGACGCGGTG
490	500	510	520	530	540
GTGTCCTTCA	GGACGGCCAT	GTGGTTCTGG	ATGACGGCGC	AGGGAAACAA	GCCGTCGTGC
550	560	570	580	590	600
CACAACGTCTG	CCCTACGCCG	CTGGACGCCG	ACGGCCGCCG	ACACCGCTGC	CGGCAGGGTA
610	620	630	640	650	660
CCCGGATACG	GAGTGATCAC	CAATATCATC	AACGGCGGGC	TCGAGTGCGG	AATGGGCCGG
670	680	690	700	710	720
AACGACGCCA	ACGTCGACCG	CATCGGCTAC	TACACGCGCT	ACTGCGGCAT	GCTCGGCACG
730	740	750	760	770	780
GCCACCGGAG	GCAACCTCGA	CTGCTACACC	CAGAGGAACT	TCGCTAGCTA	G.....

Nucleotide Sequence of cDNA corresponding to the

Amino Acid Sequence Listed as SEQ.ID. No. 2 (SEQ ID NO: 2)

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      10      20      30      40      50      60
ATGTCCACGC TGAGAGCGCG GTGTGCGACG GCCGTCCTGG CCGTCGTCCT GGCGGCGGCC

      70      80      90     100     110     120
GCGGTCACGC CGGCCACGGC CGAGCAGTGC GGCTCGCAAG CCGGCGGCGC CAAGTGCGCC

     130     140     150     160     170     180
GACTGCCTGT GCTGCAGCCA GTTCGGGTTC TCGGCGACCA CCTCGACTA CTGCGGCCCC

     190     200     210     220     230     240
CGCTGCCAGA GCCAGTGAC TGGCTGCGGT GGCGGCGGCG GCGGGGTGGC CTCCATCGTG

     250     260     270     280     290     300
TCCAGGGACC TCTTCGAGCG GTTCCTGCTC CATCGCAACG ACGCAGCGTG CCTGGCCCCG

     310     320     330     340     350     360
GGGTTCTACA CGTACGACGC CTTCTTGGCC GCCGCCGGCG CGTTCCTGGC CTTCGGCACC

     370     380     390     400     410     420
ACCGGAGACC TGGACACGCG GAAGCGGGAG GTGGCGGCCT TCTTCGGCCA GACCTCTCAC

     430     440     450     460     470     480
GAGACCACCG GCGGGTGGCC CACCGCGCCC GACGGCCCCT TCTCATGGGG CTACTGCTTC

     490     500     510     520     530     540
AAGCAGGAGC AGGGCTCGCC GCCGAGCTAC TGCGACCAGA GCGCCGACTG GCCGTGCGCA

     550     560     570     580     590     600
CCCGGCAAGC AGTACTATGG CCGCGGCCCC ATCCAGCTCA CCCACAATA CAACTACGGA

     610     620     630     640     650     660
CCGCGGGGCC GCGCAATCGG GGTGGACCTG CTGAACAATC CGGACCTGGT GGCCACGGAC

     670     680     690     700     710     720
CCGACAGTGG CGTTCAAGAC GGCGATATGG TTCTGGATGA CGACGCAGTC CAACAAGCCG

     730     740     750     760     770     780
TCGTGCCATG ACGTGATCAC GGGGCTGTGG ACTCCGACGG CCAGGGATAG CGCAGCCGGA

     790     800     810     820     830     840
CGGGTACCCG GGTATGGTGT CATCACC AAC GTCATCAACG GCGGGATCGA ATGCGGCATG

     850     860     870     880     890     900
GGGCAGAACG ACAAGGTGGC GGATCGGATC GGGTTCTACA AGCGCTATTG TGACATTTTC

     910     920     930     940     950     960
GGCATCGGCT ACGGGAATAA CCTCGACTGC TACAACCAAT TGTCGTTCAA CGTTGGGCTC

     970     980     990    1000    1010    1020
GCGGCACAGT GA.....

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Nucleotide Sequence of cDNA Corresponding to the

Amino Acid Sequence Listed as SEQ. ID. No. 3 (SEQ ID No: 8)

10	20	30	40	50	60
ATGAGAGGAG	TTGTGGTGGT	GGCCATGCTC	CCCGCGGCCT	TCGCCGTGTC	TGCGCACGCC
70	80	90	100	110	120
GAGCAATGCG	GCTCGCAGGC	CGGCGGGGCG	ACGTGCCCCA	ACTGCCTCTG	CTGCAGCAAG
130	140	150	160	170	180
TTCGGTTTCT	GCGGCACCAC	CTCCGACTAC	TGCGGCACCG	GCTGCCAGAG	CCAGTGCAAT
190	200	210	220	230	240
GGCTGCAGCG	GCGGCACCCC	GGTACCGGTA	CCGACCCCTT	CCGGCGGCGG	CGTCTCCTCC
250	260	270	280	290	300
ATTATCTCGC	AGTCGCTCTT	CGACCAGATG	CTGCTGCACC	GCAACGACGC	GGCGTGCCTG
310	320	330	340	350	360
GCCAAGGGGT	TCTACAACCTA	CGGCGCCTTC	GTCGCCGCGG	CCAACTCGTT	CTCGGGCTTC
370	380	390	400	410	420
GCGACCACAG	GTAGCACCAG	CGTCAAGAAG	CGCGAGGTGG	CCGCGTTCCT	CGCTCAGACT
430	440	450	460	470	480
TCCCACGAGA	CGACCGGCGG	GTGGCCGACG	GCGCCCGACG	GCCCCTACTC	CTGGGGCTAC
490	500	510	520	530	540
TGCTTCAACC	AGGAGCGCGG	CGCCACCTCC	GACTIONGCA	CGCCGAGCTC	GCAGTGGCCA
550	560	570	580	590	600
TGTGCGCCGG	GCAAGAAGTA	CTTCGGGCGC	GGGCCCATCC	AGATCTCACA	CAACTACAAC
610	620	630	640	650	660
TACGGGCGCG	CGGGGCAGGC	CATCGGCACC	GACCTGCTCA	ACAACCCGGA	CCTTGTGGCG
670	680	690	700	710	720
TCGGACGCGA	CCGTGTCGTT	TAAGACGGCG	TTGTGGTTCT	GGATGACGCC	GCAATCACCC
730	740	750	760	770	780
AAGCCTTCGA	GCCACGACGT	GATCACGGGC	CGGTGGAGCC	CCTCGGGCGC	CGACCAGGCG
790	800	810	820	830	840
GCGGGGAGGG	TGCCTGGGTA	CGGTGTGATC	ACCAACATCA	TCAACGGTGG	GCTCGAGTGC
850	860	870	880	890	900
GGGCGCGGGC	AGGACGGCCG	TGTCGCCGAC	CGGATCGGGT	TCTACAAGCG	CTACTGCGAC
910	920	930	940	950	960
CTCCTTGGCG	TCAGCTACGG	TGACAACCTG	GACTGCTACA	ACCAAAGGCC	GTTCGCATAG
970	980	990	1000	1010	1020
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The advantages of the present invention may be concluded as follows.

According to the present invention there are provided novel chitinase cDNAs in wheat that have different amino acid sequences from known chitinase cDNAs and confer high disease resistance in low temperature environment. Because the three chitinase cDNAs of the present invention are capable of digesting chitin at low temperature, the introduction of any one of these three different chitinase cDNAs into plants can confer plant disease resistance in low temperature environments, so that plant varieties can be provided with high resistance against psychrophilic plant pathogens such as snow molds.

While the presently preferred embodiments of the this invention have been shown and described above, it is to be understood that these disclosures are for the purpose of illustration and that various changes and modifications may be made without departing from the scope of the invention as set forth in the appended claims.